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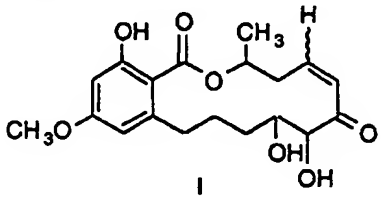
(54) Abstract Title
MEK inhibiting lactones

(57) Compounds represented by formula I



I

as well as salts and hydrates thereof have anti-cancer activity and can be used for treating ocular diseases.

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TITLE OF THE INVENTION

MEK INHIBITING COMPOUNDS, COMPOSITIONS CONTAINING SUCH COMPOUNDS AND METHODS OF TREATMENT

BACKGROUND OF THE INVENTION

The present invention relates to compounds which inhibit the phosphorylating enzyme Map/Erk kinase (MEK), compositions which contain MEK inhibiting compounds and methods of use. Additionally, the compounds are derived from a fungal culture which is included in the present invention. The organism is described herein as well as culture and fermentation techniques useful for the production of the microorganism and the compound.

The methods of treatment which are described herein relate to cancers which are effected by the Ras-activated MAP kinase cascade and to other diseases characterized by the undesirable formation of blood vessels, i.e., neoangiogenesis. The enzyme MEK phosphorylates the enzyme mitogen activated protein kinase (MAPK). By inhibiting the enzyme MEK, the Ras-activated cascade is interrupted, thus leading to a suppression of cancers in which this cascade is implicated.

Ras, Raf and the other members of this cascade are genes and proteins which are activated in a wide variety of tumors. Interrupting the Ras-cascade by inhibiting one or more of the proteins which is active in the sequence of reactions blocks the oncogenic acivity that results through over-expression or activation of one or more of the enzymes in this cascade.

MEK is a particularly desirable target in that its activation of MAPK effects a host of transcription factors and other Ser/Thr kinases. Thus, inhibiting MEK can reduce the biological effects of many other enzymes.

Neoangiogenesis occurs in conjunction with tumor growth and in certain diseases of the eye. It is characterized by excessive activity of vascular endothelial growth factor.

Vascular endothelial growth factor (VEGF) binds the high affinity membrane-spanning tyrosine kinase receptors KDR and Flt-1. Cell culture and gene knockout experiments indicate that each receptor contributes to different aspects of angiogenesis. KDR mediates the mitogenic function of VEGF whereas Flt-1 appears to modulate non-mitogenic functions such as those associated with cellular adhesion. Inhibiting KDR thus modulates the level of mitogenic VEGF activity.

Vascular growth in the retina leads to visual degeneration culminating in blindness. VEGF accounts for most of the angiogenic activity produced in or near the retina in diabetic retinopathy. Ocular VEGF mRNA and protein are elevated by conditions such as retinal vein occlusion in primates and decreased pO_2 levels in mice that lead to neovascularization. Intraocular injections of anti-VEGF monoclonal antibodies inhibit ocular neovascularization in both primate and rodent models. Regardless of the cause of induction of VEGF in human diabetic retinopathy, inhibition of ocular VEGF is useful in treating the disease.

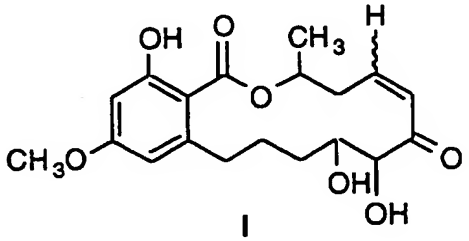
Expression of VEGF is also significantly increased in hypoxic regions of animal and human tumors adjacent to areas of necrosis. Monoclonal anti-VEGF antibodies inhibit the growth of human tumors in nude mice. Although these same tumor cells continue to express VEGF in culture, the antibodies do not diminish their mitotic rate. Thus tumor-derived VEGF does not function as an autocrine mitogenic factor. Therefore, VEGF contributes to tumor growth in vivo by promoting angiogenesis through its paracrine vascular endothelial cell chemotactic and mitogenic activities. These monoclonal antibodies also inhibit the growth of typically less well vascularized human colon cancers in athymic mice and decrease the number of tumors arising from inoculated cells. Viral expression of a VEGF-binding construct of Flk-1, the mouse KDR receptor homologue, truncated to eliminate the cytoplasmic tyrosine kinase domains but retaining a membrane anchor, virtually abolishes the growth of a transplantable glioblastoma in mice presumably by the

dominant negative mechanism of heterodimer formation with membrane spanning endothelial cell VEGF receptors. Embryonic stem cells, which normally grow as solid tumors in nude mice, do not produce detectable tumors if both VEGF alleles are knocked out. Taken together, these data indicate the role of VEGF in the growth of solid tumors. Inhibition of KDR or Flt-1 is implicated in pathological neoangiogenesis, and these are useful in the treatment of diseases in which neoangiogenesis is part of the overall pathology, e.g., diabetic retinal vascularization, as well as various forms of cancer.

Cancers which are treatable in accordance with the present invention demonstrate high levels of gene and protein expression. Examples of such cancers include cancers of the brain, genitourinary tract, lymphatic system, stomach, larynx and lung. These include histiocytic lymphoma, lung adenocarcinoma and small cell lung cancers. Additional examples include cancers in which overexpression or activation of Raf-activating oncogenes (e.g., K-ras, erb-B) is observed. More particularly, such cancers include pancreatic and breast carcinoma.

SUMMARY OF THE INVENTION

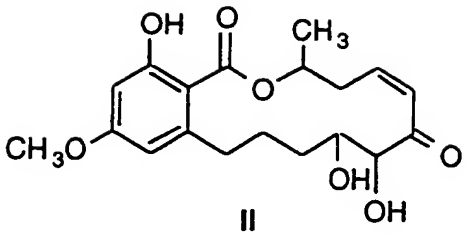
A compound is disclosed in accordance with formula I:



I

or a salt or hydrate thereof.

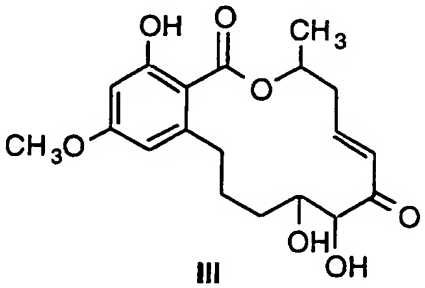
More particularly, a compound in accordance with formula II:



II

or a salt or hydrate thereof is disclosed.

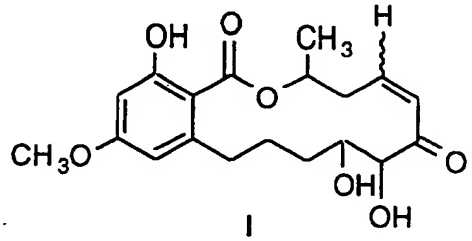
Additionally, a compound in accordance with formula III:



III

or a salt or hydrate thereof is disclosed.

Also disclosed is a pharmaceutical composition which is comprised of a compound represented by the formula I:



I

or a pharmaceutically acceptable salt or hydrate thereof in combination with a carrier.

Also included is a method of treating cancer in a mammalian patient in need of such treatment which is comprised of admininstering to said patient an anti-cancer effective amount of a

compound of formula I or a pharmaceutically acceptable salt or hydrate thereof.

Also included is a substantially pure culture of a fungal microorganism which produces a compound of formula I or a salt or hydrate thereof.

More particularly, a substantially pure culture of the organism Phoma sp. is included herein.

Even more particularly, a substantially pure culture of the organism described in accordance with ATCC No. 74403

Also included is a process for producing a compound of formula I, or a salt or hydrate thereof, which comprises aerobically cultivating a culture of ATCC No. 74403 in a nutrient medium containing assimilable sources of carbon and nitrogen and isolating said compound therefrom.

Also included in the present invention is a method of treating diseases in which neoangiogenesis is implicated, which is comprised of administering to a mammalian patient in need of such treatment a compound of formula I or a pharmaceutically acceptable salt or hydrate thereof in an amount which is effective for reducing neoangiogenesis.

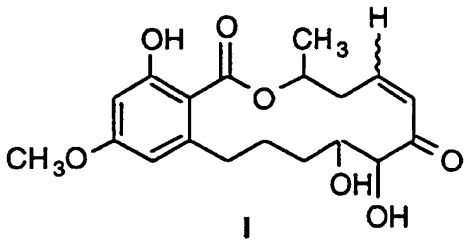
More particularly, a method of treating ocular disease in which neoangiogenesis occurs is included herein, which is comprised of administering to a mammalian patient in need of such treatment a compound of formula I or a pharmaceutically acceptable salt or hydrate thereof in an amount which is effective for treating said ocular disease.

More particularly, a method of treating retinal vascularization is included herein, which is comprised of administering to a mammalian patient in need of such treatment a compound of formula I or a pharmaceutically acceptable salt or hydrate thereof in an amount which is effective for treating retinal vascularization. Diabetic retinopathy is an example of a disease in which neoangiogenesis or retinal vascularization is part of the overall disease etiology.

These and other aspects of the invention will be apparent from the teachings contained herein.

DETAILED DESCRIPTION OF THE INVENTION

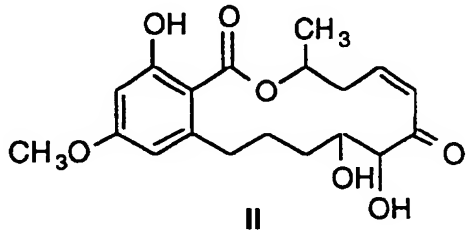
The compounds of the present invention are in accordance with formula I:



I

Salts and hydrates thereof are included.

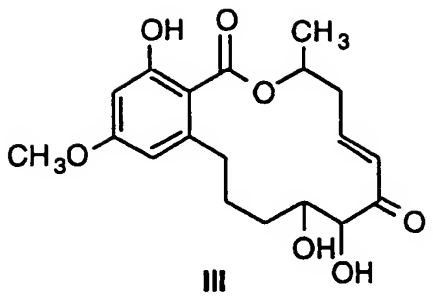
A preferred subset of compounds of the present invention is in accordance with formula II:



II

The compound has the name: [5Z]-3,4,9,10,11,12-hexahydro-8,9,16-trihydroxy-14-methoxy-3-methyl-1H-2-benzoxacyclotetradecin-1,7(8H)-dione. Salts and hydrates thereof are included. As will be appreciated, formula II represents a subset of compounds of formula I wherein the carbon-carbon double bond is in the cis configuration.

Additionally, another subset of compounds of the present invention which is of interest is in accordance with formula III:



III

Salts and hydrates thereof are included. The compound has the name: [5E]-3,4,9,10,11,12-hexahydro-8,9,16-trihydroxy-14-methoxy-3-methyl-1H-2-benzoxacyclotetradecin-1,7(8H)-dione. As will be appreciated, formula III represents a subset of compounds of formula I wherein the carbon-carbon double bond is in the trans configuration.

Also included is a pharmaceutical composition which is comprised of a compound represented by the formula I or a pharmaceutically acceptable salt or hydrate thereof in combination with a carrier.

Also included is a method of treating cancer in a mammalian patient in need of such treatment which is comprised of admininstering to said patient an anti-cancer effective amount of a compound of formula I or a pharmaceutically acceptable salt or hydrate thereof.

Also included is a substantially pure culture of a fungal microorganism which produces a compound of formula I or a salt or hydrate thereof.

More particularly, a substantially pure culture of a fungus Phoma sp. is included herein.

Even more particularly, a substantially pure culture of the fungus described in accordance with ATCC No. 74403 is included.

Also included is a process for producing a compound of formula I, or a salt or hydrate thereof, which comprises aerobically cultivating a culture of ATCC No. 74403 in a nutrient medium containing assimilable sources of carbon and nitrogen and isolating said compound therefrom.

ATCC Deposit 74403

Before the U.S. filing date of the present application, a sample of the fungus was deposited at the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852. The culture access designation is 74403. This deposit will be maintained in the ATCC for at least 30 years and will be made available to the public upon the grant of a patent disclosing it. It should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by government action.

ISOLATION OF FUNGUS

The culture was isolated from the tissues of the ascoma of the ascomycete Helvella acetabulum (Helvellaceae, Pezizales), growing under Populus sp. (poplar) in Molina de Aragón (Guadalajara, Spain). Small pieces taken from the ascoma were plated onto commeal agar (Difco) Petri dishes. The plates were incubated for several days at 22°C, and fungal colonies developing from the pieces of fungal tissue were removed and transferred to clean PDA (potato dextrose agar, Difco) plates. The PDA plates were incubated at 22°C for 2 weeks, and the pure cultures transferred to PDA slants, which were stored at 4°C until ready for use as the source for the fermentation process. From the PDA plates, the fungus was identified as a coelomycete belonging to the form-genus Phoma.

CHARACTERIZATION OF FUNGUS

Colonies grown on oatmeal agar (Difco) at 23°C, 12 hr photoperiod, growing slowly, attaining 28-30 mm diameter in 14 days, with margin appressed, even, with aerial mycelium floccose to velvety, azonate, raised, colorless at the margin, soon white to pinkish buff, Pale Pinkish Cinnamon, Light Pinkish Cinnamon, becoming gray, Neutral Gray, Dark Gull Gray at the center (capitalized color names from Ridgway, R. 1912. Color Standards and Nomenclature.

published by the author. Washington, DC), with reverse dull salmon-gray. Odors and exudates absent.

Colonies grown on YME agar (Difco malt extract 10 g, Difco yeast extract 4 g, glucose 4 g, agar 20 g, 1 L H_2O) at 23°C, 12 hr photoperiod, growing slowly, attaining 24-26 mm diameter in 14 days, appressed at the margin, raised towards the center, sulcate, granular, with scant aerial mycelium, azonate, pinkish buff at the margin, soon dark grayish brown, Light Vinaceous Cinnamon, Cinnamon-Drab to dark brown, Benzo Brown, Clove Brown, or nearly black, dark reddish brown in reverse, Walnut Brown, Warm Sepia, Bister. No growth at 37°C.

Colonies grown on commeal agar (CMA) (Difco) at 23°C, 12 hr photoperiod, attaining 28 mm in 14 days, submerged to appressed with no aerial mycelium, translucent to pale pinkish buff, Pale Pinkish Buff, with scattered reddish brown conidiomata at inoculation point. Exudates and odors absent.

Conidiomata, as observed on CMA, pycnidial, up to 400 µm in diameter, subgloblose to pyriform, papillate or not, ostiolate, gregarious, often confluent, translucent to pale reddish brown to brown, shiny, occasionally submerged, usually on or at agar surface. Conidiomata wall a textura angularis in face view, composed of densely interwoven hyphae and dark irregular plate-like cells. Conidiogenous cells enteroblastic, phialidic, hyaline, ampulliform to doliiform, with or without a short neck, with periclinal thickenings often evident at conidiogenous locus, 5-11 µm in diameter, arising directly from a pseudoparenchymatous layer lining the conidiomatal cavity.

Conidia 4-9 µm x 2-3 µm, ellipsoidal to short cylindrical, occasionally curved, hyaline, smooth, aseptate, germinating on various agar media at room temperate within 24 hrs, pale pink to vinaceous in mass. Chlamydospores not observed. Mycelium composed of highly branched, simple septate, hyaline to dematiaceous hyphae characteristic of many ascomycetous fungi.

Following the classification scheme of Sutton (Sutton, B. C. 1980. The Coelomycetes. Commonwealth Mycological Institute, Kew. U.K.), the fungus is assigned to the form genus Phoma based on the following set of characteristics: minute, non-stromatic pycnidial conidiomata; conidomata with a regular ostiole; broad dolfiiform conidiogenous cells; and hyaline, aseptate conidia.

Thus included in the present invention is a substantially pure culture of a fungal microorganism which produces a compound of formula I or a salt or hydrate thereof.

More particularly, a substantially pure culture of the fungus Phoma sp. which produces a compound of formula I or a salt or hydrate thereof is included herein.

Even more particularly, a substantially pure culture of the fungal organism deposited as ATCC No. 74403 is included herein.

Also included is a process for producing a compound of formula I which comprises aerobically cultivating a culture of ATCC No. 74403 in a nutrient medium containing assimilable sources of carbon and nitrogen and isolating said compound therefrom.

FERMENTATION PROCEDURE

In general, the fungal organism is cultured on a solid medium, or in an aqueous nutrient medium containing sources of assimilable carbon and nitrogen. For example, the cultures can be grown under submerged aerobic conditions (e.g., shaking culture, submerged culture, etc.). The desired pH may be maintained by the use of a buffer such as morpholinoethane-sulfonic acid (MES), morpholinopropanesulfonic acid (MOPS), and the like, or by choice of nutrient materials which inherently possess buffering properties.

The preferred source of carbon in the nutrient medium are carbohydrates such as glucose, xylose, galactose, glycerin, starch, dextrin, and the like. Other cources which may be included are maltose, rhamnose, raffinose, arabinose, mannose, sodium succinate, and the like.

The preferred sources of nitrogen are yeast extract, meat extract, peptone, gluten meal, cottonseed meal, soybean meal and other vegetable meals (partially or totally defatted), casein hydrolysates, soybean hydrolysates, and yeast hydrolysates, corn steep liquor, dried yeast, wheat germ, feather meal, peanut powder, distiller's solubles, etc., as well as inorganic and organic nitrogen compounds such as ammonium salts (e.g., ammonium nitrate, ammonium sulfate, ammonium phosphate, etc.), urea, amino acids, and the like.

The carbon and nitrogen sources, though advantageously employed in combination, need not be used in their pure form, because less pure materials which contain traces of growth factors and considerable quantities of mineral nutrients, are also suitable for use. When desired, there may be added to the medium mineral salts such as sodium or calcium carbonate, sodium or potassium phosphate, sodium or potassium chloride, sodium or potassium iodide, magnesium salts, copper salts, cobalt salts, and the like. If necessary, especially when the culture medium foams seriously, a defoaming agent, such as liquid paraffin, fatty oil, plant oil, polyglycol, mineral oil or silicone may be added.

As to the conditions for the production of the fungus in massive amounts, submerged aerobic cultural conditions is one method of culturing the cells. For the production in small amounts, a shaking or surface culture in a flask or bottle is employed. Furthermore, when the growth is carried out in large tanks, it is preferable to use the vegetative forms of the organism for inoculation in the production tanks in order to avoid growth lag in the process of production. Accordingly, it is desirable first to produce a vegetative inoculum of the organism by inoculating a relatively small quantity of culture medium with spores or mycelia of the organism and culturing said inoculated medium, also called the "seed medium", and then to transfer the cultured vegetative inoculum aseptically to large tanks. The fermentation medium, in which the inoculum is produced, is generally autoclaved to sterilize the medium prior to inoculation. The

pH of the medium is generally adjusted to about 6-7 prior to the autoclaving step.

Agitation and aeration of the culture mixture may be accomplished in a variety of ways. Agitation may be provided by a propeller or similar mechanical agitation equipment, by revolving or shaking the fermentor, by various pumping equipment, or by the passage of sterile air through the medium. Aeration may be effected by passing sterile air through the fermentation mixture.

The fermentation is usually conducted at a temperature between about 20°C and 30°C, preferably about 22-25°C, for a period of about 7-28 days, which may be varied according to fermentation conditions and scales.

Specific fermentation conditions for the production of compounds of formulae I, II and III are set forth below. The culture was maintained on agar plugs in vials containing sterile water stored at 4°C until ready for use. The seed culture was inoculated by aseptically transferring one agar plug into a 250 ml Erlenmeyer flask containing 50 ml seed medium of the following composition (in g/liter):

corn steep powder, 2.5
tomato paste, 40.0;
oat flour, 10.0;
glucose, 10.0 and
trace elements solution, 10 ml/liter

The Trace elements solution consisted of the following components:

$FeSO_4 \cdot 7H_2O$, 1.0 g/liter;
$MnSO_4 \cdot 4H_2O$, 1.0 g/liter;
$CuCl_2 \cdot 2H_2O$, 0.025 g/liter;
$CaCl_2 \cdot 2H_2O$, 0.1 g/liter;
H_3BO_3, 0.056 g/liter;
$(NH_4)_6MoO_{24} \cdot 4H_2O$, 0.019 g/liter;
$ZnSO_4 \cdot 7H_2O$, 0.2 g/liter;

dissolved in 0.6 N HCl.

The seed medium was prepared with distilled water. The pH can be adjusted to 6.8 by adding NaOH, and the medium dispensed into 250 ml Erlenmeyer flasks and capped with cotton plugs before being autoclaved at 121°C for 20 minutes.

Alternatively, the culture was maintained as frozen vegetative mycelia at -75°C until use, and then 1 ml used to inoculate a flask containing the seed medium above.

The seed culture was incubated at 25°C on a gyratory shaker (220 rpm, 5.1 cm throw) for 4-7 days prior to the inoculation of fermentation flasks.

One production medium was formulated as follows:

sucrose, 80.0 g/liter;
yellow corn meal, 50.0 g/liter
yeast extract, 1.0 g/liter
no pH adjustment

An alternate production medium contains:

maltose, 75.0 g/liter
V8 juice, 200 ml/liter
Soy flour, 1.0 g/liter
L-proline, 3.0 g/liter
MES buffer, 16.2 g/liter
pH to 6.5

The production media were prepared with distilled water. They were dispensed into 250 ml Erlenmeyer flasks (50.0 mls per flask) and capped with cotton plugs before being autoclaved at 121°C for 20 minutes. Fermentation flasks were inoculated with 2.0 ml vegetative seed growth and were incubated at 25°C, 85% humidity on a gyratory shaker (220 rpm, 5.1 cm throw) for 7-28 days. (When flasks were incubated in the dark, production appears to occur more

quickly and reaches a higher level, compared to incubation in the light.) This fermentation broth is frozen or extracted directly with MEK.

ISOLATION OF COMPOUNDS

A frozen broth (11 ml WBE) was warmed at room temprature until melted, extracted twice with MEK (1 : 1 v/v), filtered through a Buchner filter layered with a filter paper, and the filter cake was washed with small portions of fresh MEK. The filtrates were then pooled and the solvent was removed in vacuo. Dry crude residue was subjected to preparative TLC on SiO_2 (7.5% methanol in methylene chloride). The active fraction was then applied to reverse phase HPLC on Zorbax RX C_8 (9.4 X 25 semipreparative column) using a gradient consisted of water-acetonitrile at ambient temperature. The peak representing Compound II was pooled to give 1.6 mg.

A whole broth regrowth of the organism (2.5 liter WBE) was extracted with MEK and the solvent was removed in vacuo as described above. The dry residue of the crude MEK extract was loaded onto SiO_2 column and eluted with shallow methanol-methylene chloride stepwise gradient. The column fraction containing Compound II was dissolved in a mixture of methanol and methylene chloride; the solution was left under N_2 until milky-white precipitate started to form on the surface. The mixture was then ultrasonicated to dissolve the precipitate, and again left under N_2. The procedure was repeated several times until small amount of precipitate no longer went into solution upon ultrasonication. Aliquots of fresh methylene chloride were added until the mixture became homogeneous again, then the solution was stored in a freezer overnight. The cold mixture was filtered through a sintered glass filter; the filter cake was washed with small portions of fresh methanol a few times and dried under vacuum overnight to produce Compound II.

The mother liquor was applied to reverse phase HPLC on Zorbax RX C8 (21.2 x 25) using 25% CH_3CN-H_2O (flow rate, 10 ml/min; ambient temperature) for isolation of Compound III.

NMR/MASS SPECTRAL CHARACTERIZATION

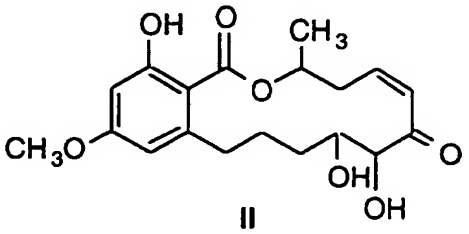
Compound II : Mass spectra were recorded on Jeol SX-102A (electron impact, EI,90eV) and on a Finnigan LCQ (LC-MS-ESI, Liquid chromatography-Electrospray ionization) mass spectrometers. Exact mass measurements were performed at high resolution (HR-EI) using perfluorokerosene (PFK) as an internal standard. The molecular ion is observed at m/z 364 by EI-MS and was determined by high resolution analysis to correspond to a molecular formula of $C_{19}H_{24}O_7$ (found 364.1530, calculated 364.1522, for M+). A critical fragment ion is observed at m/z 192 and was found to correspond to an empirical formula of $C_{11}H_{12}O_3$ (found 192.0784, calculated 192.0786).

The ^1H and ^{13}C NMR data were acquired at 25°C and referenced to the solvent peak (CD_2Cl_2) at δ5.32 and 54.0 ppm downfield of TMS, respectively.

Compound III : Mass spectra were recorded on Jeol SX-102A (electron impact, EI,90eV) and on a Finnigan LCQ (LC-MS-ESI, Liquid chromatography-Electrospray ionization) mass spectrometers. Exact mass measurements were performed at high resolution (HR-EI) using perfluorokerosene (PFK) as an internal standard. The molecular ion is observed at m/z 364 by EI-MS and was determined by high resolution analysis to correspond to a molecular formula of $C_{19}H_{24}O_7$ (found 364.1533, calculated 364.1522, for M+). A critical fragment ion is observed at m/z 192 and was found to correspond to an empirical formula of $C_{11}H_{12}O_3$ (found 192.0782, calculated 192.0786).

The ^1H and ^{13}C NMR data were acquired at 25°C and referenced to the solvent peak (CD_2Cl_2) at δ5.32 and 54.0 ppm downfield of TMS, respectively.

Based upon the Mass Spec characterization above and the NMR data provided below, Compound II was assigned the following structure:

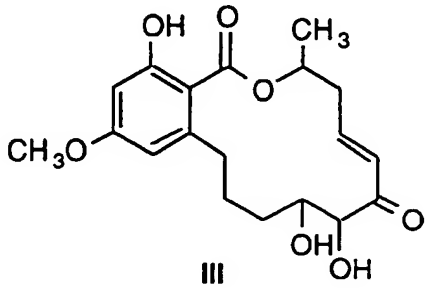


II

^{1}H NMR (CD_2Cl_2 spiked with CD_3OD, 400 MHz): δ 1.13 (1H, m), 1.39 (3H, d, J = 6.0 Hz), ~1.50 (2H, m), ~1.70 (1H, m), ~2.48 (1H, m), ~2.52 (1H, m), 2.93 (1H, ddd, J = 3.6, 12, 14.4 Hz), 3.32 (1H, dt, J = 17.6, 11.2 Hz), 3.78 (3H, s), 3.80 (1H, m), 4.48 (1H, d, J = 2.4 Hz), 5.38 (1H, m), 6.22 (1H, dt, J = 2.4, 11.6 Hz), ~6.29 (1H, obsc), 6.28 (1H, d, J = 2.8 Hz), 6.30 (1H, d, J = 2.8 Hz), 6.38 (1H, dd, J = 3.2, 11.6 Hz).

^{13}C NMR (CD_2Cl_2 spiked with CD_3OD, 100 MHz): 21.1, 28.9, 32.8, 36.9, 37.5, 55.9, 73.2, 74.1, 81.8, 99.3, 105.0, 109.6, 126.8, 146.5, 148.0, 164.8, 166.6, 172.1, 200.5 ppm.

Based upon the Mass Spec characterization described above and the NMR data shown below, Compound III was assigned the following structure:



III

^{1}H NMR (CD_2Cl_2, 400 MHz): δ ~1.35 (1H, m), 1.44 (3H, d, J = 6.8 Hz), ~1.60 (1H, m), ~1.67 (2H, m), ~2.52 (1H, m), ~2.57 (1H, m), 2.84 (1H, m), 3.09 (1H, ddd, J = 4.0, 12.0, 14.6 Hz), 3.79 (3H, s), 3.90 (1H, m), 4.64 (1H, d, J = 2.8 Hz), 5.55 (1H, m), 6.33 (2H, m), 6.40 (1H, dt, J = 16.0, 1.2), 6.97 (1H, dddd, J = 6.8, 8.0, 14.8, 16.0 Hz).

^{13}C NMR (CD_2Cl_2 spiked with CD_3OD, 100 MHz): 19.5, 27.3, 33.0, 36.4, 38.2, 55.9, 71.5, 73.4, 77.4, 99.4, 105.2, 109.8, 132.0, 143.8, 147.9, 164.8, 166.0, 171.4, 199.7 ppm.

The invention described herein includes a pharmaceutical composition which is comprised of a compound of formula I or a pharmaceutically acceptable salt or hydrate thereof in combination with a carrier. As used herein the terms "pharmaceutically acceptable salts" and "hydrates" refer to those salts and hydrated forms of the compound which would be apparent to the pharmaceutical chemist, i.e., those which favorably affect the physical or pharmacokinetic properties of the compound, such as solubility, palatability, absorption, distribution, metabolism and excretion. Other factors, more practical in nature, which are also important in the selection, are the cost of the raw materials, ease of crystallization, yield, stability, solubility, hygroscopicity and flowability of the resulting bulk drug.

When a compound of formula I is present as a salt or hydrate which is non-pharmaceutically acceptable, this can be converted to a salt or hydrate form which is pharmaceutically acceptable in accordance with the present invention.

When the compound is negatively charged, it is balanced by a counterion, e.g., an alkali metal cation such as sodium or potassium. Other suitable counterions include calcium, magnesium, zinc, ammonium, or alkylammonium cations such as tetramethylammonium, tetrabutylammonium, choline, triethylhydroammonium, meglumine, triethanolhydroammonium, etc. An appropriate number of counterions is associated with the molecule to maintain overall charge neutrality. Likewise when the

compound is positively charged, e.g., protonated, an appropriate number of negatively charged counterions is present to maintain overall charge neutrality.

Pharmaceutically acceptable salts also include acid addition salts. Thus, the compound can be used in the form of salts derived from inorganic or organic acids. Examples include acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, pamoate, pectinate, persulfate, 3-phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate and undecanoate.

As is evident from formulas II and III, various isomers are possible. All are included in the present invention.

The compounds of the invention can be formulated in a pharmaceutical composition by combining the compound with a pharmaceutically acceptable carrier. Examples of such compositions and carriers are set forth below.

The compounds may be employed in powder or crystalline form, in solution or in suspension. They may be administered orally, parenterally (intravenously or intramuscularly), topically, transdermally or by inhalation.

Thus, the carrier employed may be, for example, either a solid or liquid. Examples of solid carriers include lactose, terra alba, sucrose, talc, gelatin, agar, pectin, acacia, magnesium stearate, stearic acid and the like. Examples of liquid carriers include syrup, peanut oil, olive oil, water and the like.

Similarly, the carrier for oral use may include time delay material well known in the art, such as glyceryl monostearate or glyceryl distearate alone or with a wax.

Topical applications may be formulated in carriers such as hydrophobic or hydrophilic bases to form ointments, creams, lotions, in aqueous, oleaginous or alcoholic liquids to form paints or in dry diluents to form powders.

Examples of oral solid dosage forms include tablets, capsules, troches, lozenges and the like. The size of the dosage form will vary widely, but preferably will be from about 25 mg to about 500mg. Examples of oral liquid dosage forms include solutions, suspensions, syrups, emulsions, soft gelatin capsules and the like. Examples of injectable dosage forms include sterile injectable liquids, e.g., solutions, emulsions and suspensions. Examples of injectable solids would include powders which are reconstituted, dissolved or suspended in a liquid prior to injection.

In injectable compositions, the carrier is typically comprised of sterile water, saline or another injectable liquid, e.g., peanut oil for intramuscular injections. Also, various buffering agents, preservatives and the like can be included.

For the methods of treatment disclosed herein, dosages can be varied depending upon the overall condition of the patient, the nature of the illness being treated and other factors. An example of a suitable oral dosage range is from about 0.1 to about 80 mg/kg per day, in single or divided doses. An example of a suitable parenteral dosage range is from about 0.1 to about 80 mg/kg per day, in single or divided dosages, administered by intravenous or intramuscular injection. An example of a topical dosage range is from about 0.1 mg to about 150 mg, applied externally from about one to four times a day. An example of an inhalation dosage range is from about 0.01 mg/kg to about 1 mg/kg per day.

The compounds may be administered in conventional dosages as a single agent or in combination with other therapeutically active compounds.

Kinase inhibition is demonstrated in accordance with the following protocol.

MEK PROTEIN KINASE ASSAY

35 µl water was mixed with 5 µl sample in 50% aqueous dimethyl sulfoxide in a round bottom polyproylene plate. 5 µl of 50% aqueous dimethyl sulfoxide was used as the control for total enzymatic reaction and 5 µl of 1 M ethylenediamine-tetraacetic acid (EDTA) was used as the total inhibition control (blank). 40 µl of substrate mixture containing catalytically inactive Mapk (K-52R), Sodium Orthovanadate, MgCl2 and Hepes buffer and 20 µl of enzyme mixture containing [^{33}P]-ATP, cold ATP, bovine serum, beta -mercaptoethanol and purified cloned MEK were added separately to each assay well. The final concentration of various components are as listed below:

Components	Concentration
MEK(nM)	0.72
GST-mapk(µM)	0.35
ATP (µM)+ 1.4 µCi of 33-P-ATP	10
Hepes (mM)	25
BSA(µg/ml)	100
Beta-mercaptoethanol (mM)	2.38
MgCl2 (mM)	5
Orthovanadate (µM)	200
EDTA (mM)	0.5

The mixture is incubated at room temperature for 2 hours and the reaction is stopped with 100 µl of 125 mM EDTA and 125 mM Sodium Pyrophosphate solution.

The [^{33}P]-phosphorylated Mapk is captured by filtering through a Millipore 0.45 µM Immobilon-P Membrane which is pre-wetted with 100 µl methanol and pre-washed three times with 300 µl water. The filter plate is then washed five times with 300 µl of 20 mM Tris buffer pH 8.0. The bottom of filter is peeled off and the plate was dried. The adapter plate is placed on a filter plate and 50 µl of microscint-20™ scintillation fluid is added to each well. The

captured radioactivity is counted with a Packard Top Count™. The % inhibition is calculated as below:

$$\% \text{ INHIBITION} = \frac{(\text{CPM}_{\text{avg total binding}}) - (\text{CPM}_{\text{sample}})}{(\text{CPM}_{\text{avg total binding}}) - (\text{CPM}_{\text{avg blank}})} \times 100$$

The IC_{50} of each compound is determined by semi-log plot of the % inhibition of each compound versus the concentration of compound tested.

Anti-cancer activity is demonstrated in accordance with the following protocol.

SOFT AGAR ASSAY

Anti-cancer activity is also demonstrated using a soft agar assay, the procedure for which is set forth in Sepp-Lorenzo, et al. Cancer Research Vol. 55: 5302-5309 (1995) with the following modifications: The compound was added on top of the cell-containing agar layer as a 2 x-solution in medium rather than including it in the agar layer. Additionally, cell growth was measured after 2 weeks by an Alamar Blue mitochondrial respiration assay, in which the Alamar Blue solution was added directly on top of the agar layer.

VEGF RECEPTOR KINASE ASSAY

VEGF receptor kinase activity is measured by incorporation of radio-labeled phosphate into polyglutamic acid, tyrosine, 4:1 (pEY) substrate. The phosphorylated pEY product is trapped onto a filter membrane and the incoporation of radio-labeled phosphate quantified by scintillation counting.

MATERIALS

VEGF receptor kinase

The intracellular tyrosine kinase domains of human KDR (Terman, B.I. et al. Oncogene (1991) vol. 6, pp. 1677-1683.) and Flt-1 (Shibuya, M. et al. Oncogene (1990) vol. 5, pp. 519-524) were cloned as glutathione S-transferase (GST) gene fusion proteins. This was accomplished by cloning the cytoplasmic domain of the KDR kinase as an in frame fusion at the carboxy terminus of the GST gene. Soluble recombinant GST-kinase domain fusion proteins were expressed in Spodoptera frugiperda (Sf21) insect cells (Invitrogen) using a baculovirus expression vector (pAcG2T, Pharmingen).

Lysis buffer

50 mM Tris pH 7.4, 0.5 M NaCl, 5 mM DTT, 1 mM EDTA, 0.5% triton X-100, 10 % glycerol, 10 µg/ml of each leupeptin, pepstatin and aprotinin and 1mM phenylmethylsulfonyl fluoride (all Sigma).

Wash buffer

50 mM Tris pH 7.4, 0.5 M NaCl, 5 mM DTT, 1 mM EDTA, 0.05% triton X-100, 10 % glycerol, 10 µg/ml of each leupeptin, pepstatin and aprotinin and 1mM phenylmethylsulfonyl fluoride.

Dialysis buffer

50 mM Tris pH 7.4, 0.5 M NaCl, 5 mM DTT, 1 mM EDTA, 0.05% triton X-100, 50 % glycerol, 10 µg/ml of each leupeptin, pepstatin and aprotinin and 1mM phenylmethylsuflonyl fluoride

10 X reaction buffer

200 mM Tris, pH 7.4, 1.0 M NaCl, 50 mM $MnCl_2$, 10 mM DTT and 5 mg/ml bovine serum albumin (Sigma).

Enzyme dilution buffer

50 mM Tris, pH 7.4, 0.1 M NaCl, 1 mM DTT, 10 % glycerol, 100 mg/ml BSA.

10 X Substrate

750 mg/ml poly (glutamic acid, tyrosine; 4:1) (Sigma).

Stop solution

30% trichloroacetic acid, 0.2 M sodium pyrophosphate (both Fisher).

Wash solution

15% trichloroacetic acid, 0.2 M sodium pyrophosphate.

Filter plates

Millipore #MAFC NOB, GF/C glass fiber 96 well plate.

METHOD

A. Protein purification

1. Sf21 cells were infected with recombinant virus at a multiplicity of infection of 5 virus particles/ cell and grown at 27 °C for 48 hours.

2. All steps were performed at 4°C. Infected cells were harvested by centrifugation at 1000 X g and lysed at 4 °C for 30 minutes with 1/10 volume of lysis buffer followed by centrifugation at 100,000Xg for 1 hour. The supernatant was then passed over a glutathione Sepharose column (Pharmacia) equilibrated in lysis buffer and washed with 5 volumes of the same buffer followed by 5 volumes of wash buffer. Recombinant GST-KDR protein was eluted with wash buffer/10 mM reduced glutathione (Sigma) and dialyzed against dialysis buffer.

B. VEGF receptor kinase assay

1. Add 5 µl of inhibitor or control to the assay in 50% DMSO.

2. Add 35 µl of reaction mix containing 5 µl of 10 X reaction buffer, 5 µl 25 mM ATP/10 µCi [^{33}P]ATP (Amersham), and 5 µl 10 X substrate.

3. Start the reaction by the addition of 10 µl of KDR (25 nM) in enzyme dilution buffer.

4. Mix and incubate at room temperature for 15 minutes.

5. Stop by the addition of 50 µl stop solution.

6. Incubate for 15 minutes at 4°C.

7. Transfer a 90 µl aliquot to filter plate.

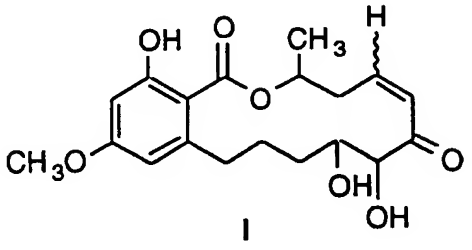
8. Aspirate and wash 3 times with wash solution.

9. Add 30 µl of scintillation cocktail, seal plate and count in a Wallac Microbeta scintillation counter.

Based upon the foregoing assay the compounds of formula I are inhibitors of VEGF and thus are useful for the inhibition of neoangiogenesis, such as in the treatment of occular disease, e.g., diabetic retinopathy and in the treatment of cancers, e.g., solid tumors.

WHAT IS CLAIMED IS:

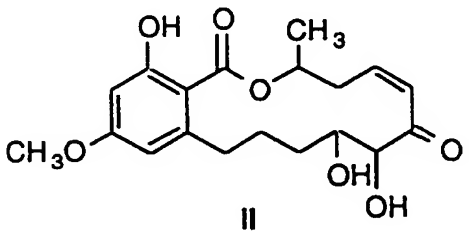
1. A compound in accordance with formula I:



I

or a salt or hydrate thereof.

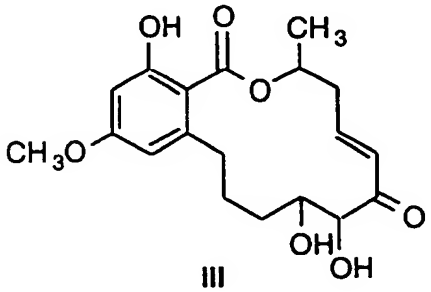
2. A compound in accordance with claim 1 of formula II:



II

or a salt or hydrate thereof.

3. A compound in accordance with claim 1 of formula III:



III

or a salt or hydrate thereof.

4. A pharmaceutical composition which is comprised of a compound in accordance with claim 1 or a pharmaceutically acceptable salt or hydrate thereof in combination with a carrier.

5. A method of treating cancer in a mammalian patient in need of such treatment which is comprised of admininstering to said patient an anti-cancer effective amount of a compound of claim 1.

6. A method of treating cancer in accordance with claim 5 wherein the cancer is selected from the group consisting of cancers of the brain, genitourinary tract, lymphatic system, stomach, larynx and lung.

7. A method in accordance with claim 5 wherein the cancer is selected from the group consisting of histiocytic lymphoma, lung adenocarcinoma, small cell lung cancers, pancreatic cancer and breast carcinoma.

8. A substantially pure culture of a fungal microorganism which produces a compound in accordance with claim 1.

9. A substantially pure culture of Phoma sp. which produces a compound in accordance with claim 1.

10. A substantially pure culture of a Phoma sp. in accordance with ATCC No. 74403 which produces a compound in accordance with claim 1.

11. A process for producing a compound of formula I or a salt or hydrate thereof, which comprises aerobically cultivating

an organism in accordance with ATCC No. 74403 in a nutrient medium containing assimilable sources of carbon and nitrogen and isolating said compound therefrom.

12. A method of treating a disease in which neoangiogenesis is implicated, which is comprised of administering to a mammalian patient in need of such treatment a compound of formula I or a pharmaceutically acceptable salt or hydrate thereof in an amount which is effective for reducing neoangiogenesis.

13. A method in accordance with claim 12 wherein the disease is an ocular disease.

14. A method of treating retinal vascularization which is comprised of administering to a mammalian patient in need of such treatment a compound of claim 1 or a pharmaceutically acceptable salt or hydrate thereof in an amount which is effective for treating retinal vascularization.

15. A method of treating diabetic which is comprised of administering to a mammalian patient in need of such treatment a compound of claim 1 or a pharmaceutically acceptable salt or hydrate thereof in an amount which is effective for treating diabetic retinopathy.

The Patent Office

28

Application No: GB 9806929.7
Claims searched: 1-15

Examiner: Peter Davey
Date of search: 4 June 1998

Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.P): C2C (CTU)

Int Cl (Ed.6): C07D 313/00

Other: Online: CAS ONLINE

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
	NONE	

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.